

Mapping and editing animal mitochondrial genomes: can we overcome the challenges?

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Abstract

The animal mitochondrial genome, although small, can have a big impact on health and disease. Non-pathogenic sequence variation among mitochondrial DNA (mtDNA) haplotypes influences traits including fertility, healthspan and longevity, whereas pathogenic mutations are linked to incurable mitochondrial diseases and other complex conditions like ageing, diabetes, cancer and neurodegeneration. However, we know very little about how mtDNA genetic variation contributes to phenotypic differences. A low frequency of recombination, the multicopy nature and nucleic acid-impenetrable membranes present significant challenges that hamper our ability to precisely map mtDNA variants responsible for traits, and to genetically modify mtDNA so that we can isolate specific mutants and characterise their biochemical and physiological consequences. Here, we summarise the past struggles and efforts in developing systems to map and edit mtDNA. We also assess the future of performing forward and reverse genetic studies on animal mitochondrial genomes.

Key words

mitochondrial DNA, genetic engineering, linkage mapping, genotype to phenotype

Introduction

Understanding how a genome instructs the phenotypic characteristics of an organism is one of the major scientific endeavours of modern molecular genetics. This is largely achieved by a combination of forward genetic studies, which use phenotypic traits to unbiasedly map the genetic basis of defined biological phenomena, and reverse genetics studies, which analyse the phenotypic effects of modifying a given genetic element. For the nuclear genome, our ability to perform forward and reverse genetics has been established for several decades [1]. The recent advances in sequencing technologies and the CRISPR-Cas9 revolution have further enhanced our capacity for mapping and editing nuclear genes with unprecedented efficiency in a multitude of organisms [2], leading to plentiful applications in research, industry, medicine and agriculture.

In contrast, the mitochondrial genome has been left behind in this genetic engineering era. Infrequent recombination and the multicopy nature of mtDNA present many challenges that prevent us from mapping the genetic underpinnings of phenotypic traits. Moreover, the lack of a robust method to genetically modify mtDNA in nearly all species leaves us little power to study various aspects of mtDNA biology and to model disease progression caused by pathogenic mutations. Given the essential functions of mtDNA and its link to incurable diseases, there is increasing pressure for the development of genetic tools to dissect the complex roles mtDNA plays in development, ageing, disease and evolution. In this article, we discuss the motivation and challenges of mapping and editing animal mtDNA.

Why do we want to link mtDNA genotypes to phenotypes?

With few exceptions, the mitochondrial genome is found in the matrix of the dynamic mitochondrial network of all eukaryotic cells in multiple copies. There is a vast diversity in mtDNA structure and composition among species of different kingdoms [3,4]. Generally speaking, yeast and plant mtDNA are large in size (e.g. ~76 to 86 kb in different *Saccharomyces cerevisiae* strains [5,6] and ~367 kb in *Arabidopsis thaliana* [7]), owing to an increased number of non-coding elements, such as introns and repeat sequences. The genome organisation and gene content is also more variable [3,8]. In contrast, most bilaterian mitochondrial genomes tend to be a compact circular molecule of less than 20 kb, carrying 37 intron-less genes encoding 13 proteins, 2 ribosomal RNAs (rRNAs) and 22 transfer RNAs (tRNAs) [9,10]. Besides the coding region with all the genes aligned one after the other, there is also a non-coding region (or control region) that contains elements important for replication and transcription [11].

Despite the simple composition of animal mtDNA, variations in its sequence can have broad and significant phenotypic consequences (figure 1a). Within a species, multiple mtDNA variants, known as haplotypes, exist. This is largely a consequence of uniparental inheritance, as different genotypes isolated in individual lineages evolve independently based on the demands from the local environment and the paired nuclear genome. Human haplotypes often differ for less than 0.3% of the 16.5 kb genome - usually fewer than 50 single nucleotide polymorphisms (SNPs) [12]. However, these minor differences have been associated with differences in longevity [13-17], spermatozoa motility [18-20], risk of multiple sclerosis [21,22], type 2 diabetes [23] and certain cancers [24,25]. Some haplotypes can even impact a repertoire of phenotypes (i.e. pleiotropism) [26]. More direct evidence that mtDNA sequence variation affects complex traits comes from studies in which numerous backcrosses were performed to generate strains that have the same nuclear background but different mitochondrial genotypes. For example, 9.3% of nuclear genes showed differential expression between males of five *Drosophila* lines that differed only by their mtDNA sequence, and one mtDNA genotype even led to male sterility [27]. In another study, mouse strains with different mitochondrial genotypes that differ for only 39 SNPs in the coding region showed differences in lifespan, insulin regulation, body weight and signs of ageing including telomere shortening, tumour

incidence and ovarian function [28]. In some cases, mismatches between mitochondrial and nuclear genome can even result in embryonic lethality [29-31].

Differences in organismal traits and physiology caused by mtDNA sequence variation could simply be a result of differences in respiratory competence, or other factors that are less well defined. For instance, impaired oogenesis and embryonic lethality results from incompatibility between a tRNA polymorphism in *Drosophila simulans* (*simw501*) mtDNA and a polymorphism in the corresponding tRNA synthetase gene in *Drosophila melanogaster* strain Oregon-R nuclear DNA [30,31]. Mutations that do not affect the function of canonical mtDNA genes can also cause differences in organismal traits and physiology. Recently, over 8 novel mitochondrial-derived peptides, encoded by small open reading frames identified in human and rodent mitochondrial genomes, have been shown to have retrograde signaling functions that lead to systemic effects [32-35]. One such peptide is MOTS-c, which is encoded within the mitochondrial 12S rRNA gene and has been shown to protect against age- and diet-induced insulin resistance [35]. Many long and small noncoding RNAs have also been shown to be encoded in mammalian mtDNA, but their mode of action is currently unknown [36-39]. Furthermore, human mtDNA-coding sequences contain binding sites for nuclear transcription factors (e.g. c-Jun and CEBPb), and this may endow regulatory potential to these sequences [40]. Therefore, mtDNA can influence animal physiology, development and ageing in complex ways beyond our current understanding. Mitochondrial genotypes often differ for multiple SNPs, and a system to map the causative SNP(s) of phenotypic variation will help elucidate the underlying mechanism of how non-pathogenic mitochondrial genetic variations attribute to organismal traits.

In addition to linking non-pathogenic sequence variations to phenotypic differences, there is also a need to study pathogenic mutations and how they cause disease. Pathogenic mtDNA variants can be inherited or acquired due to mutations or replication errors [41,42]. They often co-exist with wild-type genomes within an individual (called heteroplasmy), and their abundance can change as the mtDNA divides and segregates during development and ageing. To date, over 350 mutations in mtDNA have been reported to cause a spectrum of incurable mitochondrial diseases that affect 1 in 5,000 individuals in the UK [12,43]. Mitochondrial diseases caused by mtDNA mutations present diverse symptoms across individuals and tissues. Some pathogenic mtDNA mutations have pleiotropic effects, with different mutation loads causing different phenotypes (figure 1a). For instance, 3243A>G is a common pathogenic mutation in the *tRNA^{LEU}* gene that is associated with autism and diabetes when at low levels (~10-30% of total mtDNA) [44,45], encephalomyopathies at medium levels (50-90% of total mtDNA [46]) and perinatal lethality at high levels (>90% of total mtDNA). In addition, mtDNA mutations can have different biochemical and pathological consequences in different tissues [47] and individuals with different nuclear backgrounds or mitochondrial haplotypes. For instance, Leber Hereditary Optic Neuropathy (LOHN) is an inherited form of vision loss that has an acute onset of symptoms that usually begin in early adulthood. It is primarily due to one of the three homoplasmic mtDNA mutations (3460G>A in *ND1*, 11778G>A in *ND4* or 14484T>C in *ND6* [48-50]) that affect complex I activity, but there is an increased risk of developing LOHN for males [51,52], for those with mtDNA haplotype J [53,54] or those who smoke or have excessive alcohol consumption [55]. Understanding how the nuclear genome and environmental conditions impact penetrance will help us gain more insights on disease prevention and treatment options. However, without being able to isolate specific mitochondrial mutants and model their effects under different conditions, our knowledge on these aspects is very limited.

To be able to fully understand how mtDNA influences health and disease, systems are required that 1) can genetically unlink mitochondrial SNPs, therefore allowing separation of neutral polymorphisms from the causative SNPs; and 2) can isolate specific mitochondrial mutants for functional studies. However, multiple challenges hold us back from developing tools for mapping and editing animal mtDNA, which will be addressed in this prospective review.

What are the challenges to mapping mtDNA?

Many aspects of the mitochondrial genome and mitochondrial biology have made genetic mapping difficult. For instance, the effect of a mtDNA haplotype on a phenotype will depend strongly on the nuclear background [51,52,56-62]. However, the main challenges are infrequent recombination and the multicopy nature of mtDNA.

For nuclear genes, naturally-existing variations or mutations induced by radiation, chemical or insertional mutagenesis (e.g. transposable elements) can be used for linkage mapping. SNPs are then genetically unlinked through recombination during meiosis. Subsequently, strains with different genotypes are assayed for a particular phenotype to link the phenotype to certain SNPs. A similar forward genetic approach could not be easily applied to study mitochondrial genes (figure 1b). First, recombination is rare in animal mitochondria, if occurring at all. Most observations of mtDNA recombination are one-off events in only a handful of species with few details of the two parental genomes [63-71]. Second, as each cell contains many copies of mtDNA, it is difficult to create random mutations for linkage mapping. While mitochondrial mutations can be induced by chemicals, such as bleomycin [72-74], or in strains with reduced mtDNA polymerase proof reading capacity (known as mutator strains) [75-78], these approaches will generate heteroplasmic cells with individual genomes mutated at different loci and the genetic composition of each cell will be vastly different. Heteroplasmy prevents accurate linking of genotype to phenotype as the effects of individual variants are masked. This means that we can only use naturally-existing mitochondrial genotypes with defined impacts on a given phenotype. We then need to mix them together to generate a heteroplasmic organism, so that they can recombine. Artificial heteroplasmy is often achieved by mitochondrial transfer between two homoplasmic eggs, which could be problematic for certain species. Third, even if recombination can be induced to occur at a relatively high frequency, different recombinants from the two defined parental genomes can be generated within the same cell or organism. In this case, the functional consequences of a recombinant genome will be masked by other recombinants or the parental genomes in the same cell. Hence, we have to find ways to select for organisms carrying only one type of recombinant.

Can we develop a system to map animal mtDNA?

Despite the above complications, linkage mapping has been made possible with *Drosophila* mtDNA. In a number of lineages of a heteroplasmic setting, recombinant mitochondrial genomes were isolated due to rare spontaneous recombination. Each recombinant had a strong selective advantage over the two parental genomes, and thus reached homoplasmy after a few generations. They were used to map mtDNA sequences that give one of the parental genomes a selfish transmission advantage [69]. What is more exciting, the same study also generated a system to induce recombination and select for individuals homoplasmic for recombinant genomes (figure 1c). In this setup, cytoplasmic transfer was performed to generate heteroplasmic fruit flies containing two parental genomes. This was followed by expression of mitochondrially targeted restriction enzyme(s) (mitoREs) to cut the parental genomes at different positions [69]. The double strand break in one genome was efficiently repaired based on the homologous sequences presented in the other genome, and this generated a recombinant mtDNA that lacks recognition sites for the mitoREs. Use of mitoREs also selects against the parental genomes as they are linearized and degraded, whereas the recombinant mtDNA will be resistant to cutting. This system is very efficient for isolating recombinants, even if the two parental genomes are highly diverged, including genomes from different species where the sequence homology is less than ~92%. Isolation of homoplasmic recombinant genomes in this way opens up the exciting possibility of precisely defining trait-associated mtDNA SNPs.

There is no doubt much will be learnt from mapping *Drosophila* mtDNA, and a similar mitoRE system could also be applied to other animals. However, much optimisation is required to increase the

flexibility and efficiency of mtDNA mapping. The availability and location of recognition sites presented in the two parental genomes limits the mitoRE system by constraining which genomes can be studied and where crossovers will occur. This shortfall may be rescued by using other mitochondrially targeted nucleases (mito-nucleases) that can be engineered to target more sequences of choice. Mitochondrially targeted zinc finger nucleases (mitoZFNs) and transcription activator-like effector nucleases (mitoTALENs) consist of a customisable DNA binding domain fused with a nuclease domain and a mitochondrial localisation signal. In mitoZFNs, each zinc finger domain recognises a 3 or 4 nucleotide sequence and several domains can be engineered in an array to target longer sequences [79-81]. mitoTALENs provide increased sequence targeting flexibility as each transcription activator-like effector consist of an array of 34 amino acid repeats that each bind a single DNA base and can be engineered to target almost any sequence [82-88]. mitoZFNs and mitoTALENs have been used to eliminate pathogenic mtDNA that differs from the co-existing wild-type genome by only a single point mutation in rodent germ cells [86] and somatic tissues [89-91], and in patient-derived cells [87,92]. Therefore, it is feasible that they can replace mitoREs to achieve more flexible mapping in *Drosophila* and other animal models.

Of note, mitoZFNs/mitoTALENs can be difficult to implement. The importing efficiency of mitoTALENs/mitoZFNs into mitochondria can vary depending on the targeted sequence, which determines the protein properties of the DNA binding domain [93]. Since mitoZFNs/mitoTALENs work as heterodimers, both monomers need to be present in a sufficient amount inside mitochondria to function. This can be challenging if the monomers have different importing efficiencies. Furthermore, being heterodimers requires the design of two independent DNA-binding modules to target a single sequence [93]. Hence, the plasmid constructs are usually large, which can impede their *in vivo* delivery. To overcome some of these shortfalls, ZF and TALE-targeted monomeric nucleases have recently been developed [94,95] and tested in mitochondria [96]. With a smaller construct and simplification of importing only one type of DNA binding domain into mitochondria, the monomeric versions present a promising alternative that allow more efficient delivery and increased flexibility of target sequences [96].

In addition, to achieve efficient mtDNA mapping, future research in species where there is active mitochondrial recombination may help us develop ways to increase recombination frequency in animal mitochondria. For instance, key components for recombination, including RecA homologs in *A. thaliana* and Rad52-like proteins in *S. cerevisiae*, have been identified [97-100]. These and other supplementary components can be targeted to animal mitochondria to induce recombination. Proteins mediating recombination in bacteria, bacteriophages and even metazoan nuclear genomes can also be targeted to achieve the same aim.

What are the challenges to editing animal mtDNA?

Whilst mapping enables detangling of the functional consequences of individual SNPs, mtDNA editing is required to verify mapping by generating specific mutations. To date, a transformation system for mtDNA editing has been established in *S. cerevisiae* (baker's yeast) [101,102] and *Chlamydomonas reinhardtii* (green algae) [103]. Both are unicellular organisms with active recombination in mitochondria, allowing desired changes in a donor template to be introduced into the genome. Delivery of donor DNA was accomplished using biolistic bombardment into mitochondria of respiration-defective mutant strains, followed by selection of successful transformants based on respiratory function. In yeast, integration of *ARG8^m* has also been used to select for transformed cells with a mutated nuclear *ARG8* [104]. *ARG8* is required for arginine biosynthesis, so the selection is independent of respiratory function. Although the delivery of donor DNA is inefficient, and a large starting population is required to select transformants, the transformation system has made the two species very tractable models to study mitochondrial dynamics and the physiological consequences of mitochondrial mutations. For example, yeast mutants have been used to model human pathogenic

mtDNA mutations [105-108]. Integration of *ARG8^m* has been used to disrupt mitochondrial genes and to study mitochondrial gene expression [104,109,110]. Moreover, visible reporters have been inserted into the yeast mtDNA, including GFP added after the start of the *mt:Cox3* gene to study mitochondrial gene expression [111] and adaptation of the LacO-LacI-GFP system to visualize mtDNA [112]. Similarly, expression of GFP or the zeomycin resistance gene *ble* from mtDNA has been achieved in *C. reinhardtii* [113,114].

In animals, mitoREs have been expressed in the germline to isolate inheritable homoplasmic mtDNA mutants in *Drosophila* [115]. This method relies on selection of pre-existing mtDNA variants that lack the recognition site of the expressed mitoRE. The isolated *mt:ND2* and *mt:Cox1* mutants have been useful for disease modeling [116,117]. Several groups have also used them to study how transmission of co-existing mitochondrial genomes is influenced by selection [118]. However, this is the only approach that allows isolation of homoplasmic mitochondrial mutants in a metazoan, and the site of mutations is restricted to sequences proximal to or at the recognition site of the restriction enzyme used.

A transformation system to edit animal mtDNA in a more desired manner has not yet been established, and the challenges come in multiple ways (figure 1d). First, transformation of the germline mtDNA is required to edit mitochondrial genomes of multicellular animals, which scales up the difficulty of this endeavor. Moreover, in yeast and algae, mtDNA loss is not lethal because this can be rescued by supplementary factors or compensatory cellular mechanisms (e.g. glycolysis or photosynthesis). However, in animals, we cannot isolate mtDNA mutants that are homoplasmic lethal at the organismal level. A system to isolate and maintain lethal mutations, for example by expression of nuclear-encoded version of mitochondrial proteins, is far from being established in animals. Second, the rarity of recombination in animal mitochondria impedes the exchange between a donor DNA template and the endogenous genome, which is key to introduce specific mutations and precise tagging. Third, there is no efficient method to deliver donor DNA into mitochondria. There has been a great abundance of research on this topic with many exciting reports, including electroporation, protein-DNA conjugates, bacterial conjugation and nanocarriers like MITO-porter and adeno-associated virus-mediated transfer [119-130]. Nevertheless, few, if any, of these methods have been reproduced by independent laboratories, even at the cell level. One reason for this may be that it is very difficult to test for mitochondrial import definitively [131]. Most studies rely on sub-fractionation and the generation of mitoplasts (isolated mitochondria with outer membrane removed) to show uptake into the matrix. However, such methods are vulnerable to false positives caused by contamination. Success in delivery of foreign genetic materials can be verified if there is a strong selection in favour of the transformed genome. Therefore, the fourth challenge is the lack of ways to select for transformants. Successful transformation often only occurs to a very small population, so the ability to select for homoplasmic transformants is incredibly important.

Can we edit animal mtDNA in the near future?

Whilst we currently have no system to edit animal mtDNA, advances in mito-nucleases have helped solve some of the challenges we mentioned above. For example, mitoREs have been used to induce recombination [69]. Importing recombination machinery from other species into animal mitochondria (see our discussion earlier) might also increase the basal recombination frequency. Alternatively, *in vitro* modified mtDNA could be directly delivered, so that recombination is not required to incorporate specific alterations. For instance, the first transformation of algae used mtDNA purified from *C. reinhardtii* or *Chlamydomonas smithii* [132].

The challenge of selection for transformants obtained by either recombination-based repair or delivery of *in vitro* modified mtDNA could also be overcome by using mito-nucleases, which can be engineered to cut the endogenous genome to cause their subsequent degradation. In such systems,

one can construct the donor template or modified genome to lack the recognition site, so that any transformants will be resistant to the cut and thus be selected for. For example, mitoREs have already been used to create *D. melanogaster* flies homoplasmic for very diverged *Drosophila yakuba* mtDNA by eliminating the endogenous genomes after mitochondrial transfer [133]. Similarly, engineered mitoTALENs and mitoZFNs are effective at selecting against mutant genomes that differ from the co-existing wild-type genome by just one nucleotide [86,87,89,90,92]. Therefore, mito-nucleases represent powerful tools to selectively eliminate untransformed genomes.

Whilst the use of mito-nucleases enables screening of many embryos for successful mtDNA manipulation in species like *Drosophila*, it will be difficult and expensive to implement this in other animals like rodents. Editing mtDNA for these species can be first considered in cultured cell lines. A cell model will also be feasible for creating mutants that would otherwise be homoplasmic lethal at the organismal or tissue level. For instance, lethality due to reduced respiratory function could be overcome by additional supplements in the cell culture medium. In this case, cells completely lacking mtDNA (p^0 cells) can be utilised to protect the introduced *in vitro* modified mtDNA from competition with endogenous genomes.

Overall, some of the challenges holding back animal mtDNA editing may be overcome using mito-nucleases. However, not being able to reliably deliver nucleic acids into animal mitochondria still presents as a huge barrier. This prevents us from importing not only donor template or *in vitro* modified mtDNA for transformation, but also RNA for CRISPR-mediated mtDNA editing [134,135]. A mitochondria-adapted CRISPR-Cas9 platform, if established, could prompt a revolution in mitochondrial genome engineering and our biological understanding of mitochondria and mtDNA.

Concluding remarks

Significant obstacles must be overcome to achieve forward and reverse genetic studies for mitochondrial genomes. To date, there has been only one published case where homoplasmic recombinants were successfully isolated and used for functional mapping [69]. Moreover, no mtDNA transformation system has been established in any metazoan. These shortfalls hinder our understanding of how mtDNA impacts health and disease. However, there are exciting new possibilities to induce and select for mtDNA recombinants using mito-nucleases. As we learn more about mtDNA repair mechanism and mitochondrial nucleic acid import strategies, other advances are yet to come to allow us to pass through the current technical bottlenecks and revolutionize mtDNA engineering.

Acknowledgement

This work is funded by Wellcome Trust grant 203767/Z/16/Z to AK and 202269/Z/16/Z to HM.

Figure legend

Figure 1: Motivation, challenges and opportunities for mapping and editing animal mtDNA.

A) Animal mtDNA can impact health and disease. Sequence variation in mtDNA haplotypes is linked to phenotypic variation. The level of pathogenic mtDNA mutations determines the pathogenicity and severity of symptoms. **B)** Mapping of animal mtDNA can be achieved by mixing two mtDNA genotypes with defined phenotypic differences to generate heteroplasmic individuals. Homoplasmic recombinant genomes are then isolated based on spontaneous or induced recombination. Subsequently, individuals carrying different recombinant genomes are assayed for a given phenotype to define trait-associated SNP(s). Current challenges holding back our mapping capacity is the low rate of recombination in animal mitochondria and lack of a system to isolate and select individuals that are homoplasmic for only one type of recombinant genome. **C)** Expression of mito-nucleases in heteroplasmic lines can be used to induce and isolate organisms that are homoplasmic for a certain recombinant mtDNA. Expression of chosen mito-nucleases (e.g. mitoRE, mitoTALEN and mitoZFN) introduces double strand break(s) at different positions of the two parental genomes. The break in each genome will be repaired based on the homologous sequence in the other genome, resulting in the generation of recombinant genomes lacking recognition sites of the targeted nucleases. The mito-nucleases also select against the parental genomes to allow the recombinant mtDNA to take over. The black stop symbol indicates the lack of a recognition site for the expressed mito-nucleases. **D)** Multiple challenges remain in order to transform animal mtDNA, including the delivery of external DNA into mitochondria, the low frequency of recombination and the inability to select for transformed genomes. The latter two challenges may be overcome by expression of mito-nucleases, which induces recombination to promote the incorporation of the desired modification(s) into the donor template, and selects against the parental genomes to allow the takeover by the transformant.

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